UNIVERSITI PUTRA MALAYSIA

PRODUCTION OF SOLVENT (ACETONE-BUTANOL-ETHANOL) IN BTACH AND CONTINUOUS FERMENTATION BY CLOSTRIDIUM SACCHAROBUTYLICUM DSM 13864 USING GELATINISED SAGO STARCH AS SUBSTRATE

LIEW SHIAU TSUEY.

IB 2005 9

PRODUCTION OF SOLVENT (ACETONE- BUTANOL-ETHANOL) IN BATCH AND CONTINUOUS FERMENTATION BY *CLOSTRIDIUM SACCHAROBUTYLICUM* DSM 13864 USING GELATINISED SAGO STARCH AS SUBSTRATE

By

LIEW SHIAU TSUEY

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2005

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

PRODUCTION OF SOLVENT (ACETONE- BUTANOL-ETHANOL) IN BATCH AND CONTINUOUS FERMENTATION BY *CLOSTRIDIUM SACCHAROBUTYLICUM* DSM 13864 USING GELATINISED SAGO STARCH AS SUBSTRATE

By

LIEW SHIAU TSUEY

May 2005

Chairman: **Professor Arbakariya Bin Ariff, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

Study on the feasibility of using improved computer-controlled HPLC and GC systems was carried out to simplify the analysis method used in solvent fermentation. The use of HPLC system with a single injection to analyse the composition of culture broth (substrates and products) during solvent fermentation was achieved by raising the column temperature to 80°C. Although good separation of the components in the mixture was achieved, a slight peak overlapped for butyric acid and acetone was observed. However, improved GC system was developed and capable to measure the products of solvent fermentation (acetone, butanol, ethanol, acetic acid and butyric acid) within 22 min of analysis time. In order to obtain accurate quantification, GC was used to determine the products whereas HPLC was used to detect the substrates.

The effect of different sago starch concentrations on solvent fermentation by *Clostridium saccharobutylicum* DSM 13864 was studied in anaerobic condition

using 250 mL schott Duran bottle. The optimal sago starch concentration obtained was 50 g/L where the total solvent concentration, total solvent yield and total solvent productivity were 8.97 g/L, 0.20 g/g and 0.14 g/L.h, respectively. The performance of solvent fermentation was greatly improved when 2 L stirred tank fermenter was applied using 50 g/L sago starch. The fermentation time to reach the maximum total solvent concentration was shortened from 66 h to 28 h. The total solvent concentration, total solvent yield and total solvent productivity obtained was 10.89 g/L, 0.24 g/g and 0.39 g/L.h, respectively.

The total solvent production in 2 L stirred tank fermenter was significantly improved when glycerol was added to the medium. With the addition of 2 to 10 g/L glycerol to the medium, the production of total solvent was increased by about 3% to 50.4% as compared to fermentation without the addition of glycerol (10.89 g/L), respectively. Although there was a reduction in ethanol production, the production of acetone and butanol was significantly increased. Glycerol with concentration of 6 g/L was optimal for improvement of the total solvent production (16.38 g/L), total solvent yield (0.35 g/g) and total solvent productivity (0.59 g/L.h).

From the study it was found that the condition could be adjusted to suit for acids production (high dilution rate and high pH) or solvent production (low dilution rate and low pH) by manipulating the dilution rate and culture pH of single stage continuous fermentation. The highest solvent concentration in outflow (9.10 g/L) was obtained at pH 4.5 and dilution rate of 0.05 h^{-1}, which gave the overall productivity of 0.46 g/L.h. However, the highest total solvent productivity (0.85 g/L.h) was obtained at dilution rate of 0.11 h^{-1} at pH 4.5. Although the total solvent

productivity was greatly increased in continuous culture, the final solvent concentration attained in outflow was decreased by about 53% as compared to batch culture.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN PELARUT (ASETON-BUTANOL-ETANOL) SECARA FERMENTASI SEKELOMPOK DAN SELANJAR OLEH *CLOSTRIDIUM SACCHAROBUTYLICUM* DSM 13864 DENGAN MENGGUNAKAN KANJI SAGU TERGELATIN SEBAGAI SUBSTRAT

Oleh

Liew Shiau Tsuey

Mei 2005

Pengerusi: **Profesor Arbakariya Bin Ariff, PhD**

Fakulti: **Bioteknologi dan Sains Biomolekul**

Kajian feasibiliti berasaskan kemajuan Kromatografi Cecair Prestasi Tinggi (HPLC) kawalan berkomputer dan sistem Kromatografi Gas (GC) telah dijalankan untuk memudahkan kaedah analisis pelarut dalam proses fermentasi. Dalam kajian Kromatografi Gas, suntikan tunggal untuk menganalisis kesemua komponen (substrat dan produk) telah dicapai dengan meningkatkan suhu kolum kepada 80°C. Walau bagaimanapun, puncak pertindihan telah didapati bagi asid butirik dan aseton. Kaedah analisis bagi GC telah berjaya dimajukan dan berupaya untuk menganalisis produk fermentasi iaitu aseton, butanol, etanol, asid asetik dan asid butirik dalam masa 22 minit. Untuk memperolehi analisis yang tepat, produk fermentasi akan dianalisis menggunakan GC manakala substrat akan ditentukan dengan HPLC.

Kesan pelbagai kepekatan sagu kanji terhadap fermentasi pelarut menggunakan *Clostridium saccharobutylicum* DSM 13864 telah dikaji dalam botol schott Duran

berisipadu 250 mL di bawah keadaan anaerobik. Kepekatan sagu kanji yang optimum adalah 50 g/L di mana jumlah kepekatan pelarut, angkali hasil pelarut berasaskan sumber karbon digunakan, dan jumlah produktiviti pelarut adalah 8.97 g/L, 0.20 g/g dan 0.14 g/L.j masing-masing. Prestasi fermentasi pelarut dalam 50 g/L sagu kanji telah ditingkatkan apabila fermenter tangki pengaduk yang berisipadu 2 L digunakan. Masa fermentasi untuk mencapai kepekatan pelarut tertinggi telah dikurangkan dari 66 j kepada 28 j. Nilai untuk kepekatan pelarut, angkali hasil pelarut berasaskan sumber karbon digunakan dan jumlah produktiviti pelarut adalah 10.89 g/L, 0.24 g/g dan 0.39 g/L.j masing-masing.

Penambahan gliserol ke dalam medium telah meningkatkan penghasilan pelarut di dalam fermenter tangki pengaduk berisipadu 2 L. Penambahan sebanyak 2 g/L-10 g/L gliserol telah meningkatkan 3% -50.4% kepekatan pelarut berbanding dengan fermentasi tanpa penambahan gliserol (10.89 g/L). Walaupun terdapat pengurangan dari segi penghasilan etanol, tetapi peningkatan penghasilan aseton dan butanol adalah signifikan. Kepekatan 6 g/L gliserol merupakan kepekatan optimum dalam peningkatan nilai kepekatan pelarut (16.38 g/L), angkali hasil pelarut berasaskan sumber karbon digunakan (0.35 g/g) dan jumlah produktiviti pelarut (0.59 g/L.j).

Dalam kajian kultur selanjar, didapati keadaan fermentasi boleh diubah kepada penghasilan asid (kadar pencairan tinggi dan pH tinggi) ataupun penghasilan pelarut (kadar pencairan rendah dan pH rendah) dengan memanipulasi kadar pencairan dan pH. Kepekatan pelarut tertinggi pada aliran keluar (9.10 g/L) dengan jumlah produktiviti pelarut sebanyak 0.46 g/L.j telah dicapai pada pH 4.5 dan kadar pencairan 0.05 j^{-1}. Nilai jumlah produktiviti pelarut tertinggi (0.85 g/L.j) telah

dicapai pada kadar pencairan sebanyak 0.11 j^{-1} dan pH 4.5. Walaupun jumlah produktiviti pelarut telah ditingkatkan secara drastik dalam kultur selanjar tetapi kepekatan pelarut yang dicapai pada aliran keluar adalah 53% lebih rendah berbanding dengan kultur sekelompok.

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation and thankfulness to the supervisory committee members of my project, Professor Dr. Arbakariya Ariff, Associate Professor Dr. Raha Abdul Rahim and Dr. Rosfarizan Mohamad for their guidance, support and encouragement.

Special thanks to staff and friends of Fermentation Technology Unit, Laboratory of Enzyme and Microbial Technology for their guidance and assistance throughout the days in lab. Finally, I would like to express my highest gratitude to my family for their continuous support and endless love.

I certify that an Examination Committee met on 5th May 2005 to conduct the final examination of Liew Shiau Tsuey on her Master of Science thesis entitled "Production of Solvent (Acetone-Butanol-Ethanol) in Batch and Continuous Fermentation by *Clostridium saccharobutylicum* DSM 13864 Using Gelatinised Sago Starch as Substrate" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

Tey Beng Ti, PhD
Lecturer
Faculty of Engineering
Universiti Putra Malaysia
(Chairman)

Ling Tau Chuan, PhD
Lecturer
Faculty of Engineering
Universiti Putra Malaysia
(Internal Examiner)

Suraini Abd Aziz, PhD
Associate Professor
Institute of Bioscience
Universiti Putra Malaysia
(Internal Examiner)

Kopli Bujang, PhD
Associate Professor
Faculty of Resource and Science Technology
Universiti Sarawak Malaysia
(External Examiner)

GULAM RUSUL RAHMAT ALI, PhD
Professor/Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

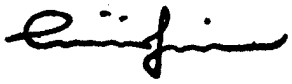
Date: 21 JUL 2005

This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the supervisory Committee are as follows:

Arbakariya Ariff, PhD
Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Chairman)

Raha Abdul Rahim, PhD
Associate Professor
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

Rosfarizan Mohamad, PhD
Lecturer
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)



AINI IDERIS, PhD
Professor/ Dean
School of Graduate Study
Universiti Putra Malaysia

Date: 11 AUG 2005

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or cocurrently submitted for any other degree at UPM or other institutions.

LIEW SHIAU TSUEY

Date: 18-7-2005

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABE	acetone-butanol-ethanol
rpm	rotation per minute
μ_{max}	maximum specific growth rate (h^{-1})
X_m	maximum cell concentration (g/L)
P_m	maximum total solvent concentration (g/L)
$Y_{p/x}$	total solvent yield (g/g)
P_r	total solvent productivity (g/L.h)

CHAPTER 1

INTRODUCTION

The anaerobic fermentation of carbohydrates to solvent (acetone-butanol-ethanol/ ABE) by *Clostridium* spesies has been well documented and commercially applied for several decades World War I and II. However, with the advent of cheaper petrochemical-based production of solvent, the production through fermentation process becomes economically unattractive and unfavorable during 1960s and, as a result, almost all the industry-scale fermentation facilities have been closed (Jones and Woods, 1986; Jones, 2001).

The quantities of acetone, butanol and ethanol produced worldwide are extremely large and almost all production is via petrochemical synthesis. As a result, the price of these products are to a large extent dependent on the price of crude oil and prices can fluctuate considerably on the open market from year to year (Jones, 2001). In addition, known crude oil reserves could be depleted in less than 50 years at the present rate of consumption (Crabbe *et al.*, 2001). According to Petronas president and chief executive officer Tan Sri Mohamad Hassan Marican, Malaysia's oil reserves were expected to last another 18 years (Lee and Kamiso, 2004). Thus, this situation represents an opportunity for the fermentation-derived process technology based on an alternative feedstock whose supply is not limited, i.e., renewable resources (biomass).

The acetone, butanol and ethanol have many commercial applications in various industries. These solvents have been used as a chemical feedstock and liquid fuels

since 1940s. During World War I, the fermentation was first aimed at the production of acetone for the manufacture of munitions by the British army and later at the production of butanol for the manufacture of lacquer in automobile industry (Jones and Woods, 1986). ABE fermentation process has remained of interest due to its potential application in biotechnology and, thus attempting to improve the production is still being intensively studied worldwide (Ishizaki *et al.*, 1999; Jones, 2001; Ezeji *et al.*, 2004).

In recent years, considerable work has been conducted towards the improvement of the traditional batch fermentation process and the development of some novel fermentation technologies. One of the problems hindering commercial development of ABE fermentation is the fact that it suffers severely from product inhibition caused principally by butanol. Therefore, various type of fermentation mode integrated with product removal system such as pervaporation, adsoprtion, liquid-liquid extraction, perstraction and gas stripping has been reported (Qureshi *et al.*, 2001). Besides, the immobilization of *Clostridium* strain using different types of immobilization support as an approach to enhance solvent production has also been well established. Cell recycling using utrafiltration was also demonstrated as a successful method for retaining biomass and increasing productivity in solvent fermentation (Jones, 2001). Another possible approaches to improve solvent production is the development of strain that manipulated at the genetic level (Dürre, 1998).

In order to reintroduce an economically competitive biological process, the major drawbacks that must be overcome first, is the high cost of the substrate. About 60%

of the overall production cost is the cost of substrate (Jones and Woods, 1986). Since solventogenic *Clostridia* are able to utilise a wide range of carbohydrate substrates, considerable research into the use of substrates cheaper than molasses (the traditional substrate for ABE production) such as potato (Linden *et al.*, 1985), corn, maize (McNeil and Kristiansen, 1986), jerusalem artichoke (Marchal *et al.*,1985), whey permeate (Ennis and Maddox, 1985), apple pomace (Voget *et al.*, 1985), peat (Forsberg *et al.*, 1986), potato waste (Grobben *et al.*, 1993), palm oil mill effluent (Lee *et al.*,1995) and domestic organic waste (López-Contreras *et al.*, 2000) have been reported.

Malaysia has a lot of potential substrate that has not been exploited for its usage as substrate for fermentation for example sago starch. Recently, some works on direct fermentation of sago starch to solvent by *C. saccharobutylicum* (formerly known as *C. acetobutylicum)* P262 using batch culture and the ability of this bacterium to secrete amylolytic enzymes have been reported by Madihah *et al.* (2001a and b), however, the performance of the process is still not acceptable for industrial application. Thus, further improvement of the process either through microbiology or engineering approach is required.

Higher level of intracellular ATP and NADH obtained in *C. butyricum* that grown on glycerol-glucose mixture was reported (Saint-Amans *et al.*, 2001). The increased level of ATP and NADH are assiociated with increased solvent production in *Clostridium acetobutylicum* (Meyer and Papoutsakis, 1989). Therefore, the feasibility of adding glycerol into the medium might be employed in order to

improve the performance of direct fermentation of sago starch to solvent using *C. saccharobutylicum* DSM 13864.

The use of fermentation technique such as continuous culture for the improvement of solvent fermentation has been well established. However, the performance of direct fermentation of sago starch to solvent using continuous culture has not been reported elsewhere. In general, most of the reports on continuous solvent fermentation focused on the use of glucose as a carbon source (Bahl *et al.*, 1982a; Monot and Engasser, 1983a; Afschar *et al.*, 1985; Fick *et al.*, 1985; Soni *et al.*, 1987a; Mollah and Stuckey, 1992).

The objectives of this study are,

i. To improve and simplify the method based on gas chromatography and high performance liquid chromatography for rapid quantification of substrates, intermediate acids and solvent produced during ABE fermentation using sago starch as a substrate.

ii. To study the effect of different sago starch concentrations on the performance of ABE fermentation using *C. saccharobutylicum* DSM 13864.

iii. To investigate the effect of the addition of glycerol into culture on the production of ABE by *C. saccharobutylicum* DSM 13864 using sago starch as a substrate.

iv. To investigate the effect of dilution rate and culture pH on ABE production in a single stage continuous fermentation of *C. saccharobutylicum* DSM 13864.

CHAPTER 2

LITERATURE REVIEW

2.1 Application of Acetone, Butanol and Ethanol (ABE)

The solvent (ABE) has a great commercial value and has been used in various industries such as for fuel, reagents, feedstock and antibiotics (Badr *et al.*, 2001). During World War I acetone was widely used in aircraft wing dopes as a fuel. Besides, smokeless powder produced from acetone has been used by British Army as the ingredient in muniations manufacture (Jones and Woods, 1986). Acetone is a good solvent and important organic raw material, mainly used to produce plexyglass, phenolics, acetic acid fiber, epoxy in chemical application field and to produce antibiotic, hormone and vitamin in pharmaceutical industry (Beijing Yanshan Petrochemical, 2004).

Butanol is used primarily in the manufacture of lacquers, rayon, detergents, brake fluid and amine additive. In addition, butanol has also been applied to chemical industry as a general solvent for fats, waxes, resins, shellac and varnish (Linden *et al.* 1985). Butanol has many characteristics, which make it a better fuel extender and now is used in the formulation of gasohol (Mollah and Stuckey, 1993) and gasoline additive (Park *et al.*, 1989). Butanol has potential to be used as a cosurfactant and enhance the release of oil from the underground water (Krouwel *et al.*, 1982).